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Neurovirulence and Immunogenicity of Attenuated Recombinant Vesicular Stomatitis Viruses in Nonhuman Primates

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ABSTRACT

In previous work, a prototypic recombinant vesicular stomatitis virus Indiana serotype (rVSIV) vector expressing simian immunodeficiency virus (SIV) gag and human immunodeficiency virus type 1 (HIV-1) env antigens protected nonhuman primates (NHPs) from disease following challenge with an HIV-1/SIV recombinant (SHIV). However, when tested in a stringent NHP neurovirulence (NV) model, this vector was not adequately attenuated for clinical evaluation. For the work described here, the prototypic rVSIV vector was attenuated by combining specific G protein truncations with either N gene translocations or mutations (M33A and M51A) that ablate expression of subgenic M polypeptides, by incorporation of temperature-sensitive mutations in the N and L genes, and by deletion of the VSIV G gene to generate a replicon that is dependent on *trans* expression of G protein for *in vitro* propagation. When evaluated in a series of NHP NV studies, these attenuated rVSIV variants caused no clinical disease and demonstrated a very significant reduction in neuropathology compared to wild-type VSIV and the prototypic rVSIV vaccine vector. In spite of greatly increased *in vivo* attenuation, some of the rVSIV vectors elicited cell-mediated immune responses that were similar in magnitude to those induced by the much more virulent prototypic vector. These data demonstrate novel approaches to the rational attenuation of VSIV NV while retaining vector immunogenicity and have led to identification of an rVSIV N4CT1gag1 vaccine vector that has now successfully completed phase I clinical evaluation.

IMPORTANCE

The work described in this article demonstrates a rational approach to the attenuation of vesicular stomatitis virus neurovirulence. The major attenuation strategy described here will be most likely applicable to other members of the *Rhabdoviridae* and possibly other families of nonsegmented negative-strand RNA viruses. These studies have also enabled the identification of an attenuated, replication-competent rVSIV vector that has successfully undergone its first clinical evaluation in humans. Therefore, these studies represent a major milestone in the development of attenuated rVSIV, and likely other vesiculoviruses, as a new vaccine platform(s) for use in humans.

In nature, vesicular stomatitis virus (VSV) is found only in the Americas. Biting insects appear to be the major vector for VSV (1, 2), infecting livestock and causing vesicular lesions at bite sites around the mouth, nose, and teats and coronary bands on the hooves. The lesions may result in lameness and weight loss due to difficulty in feeding, but they typically resolve in 7 to 10 days without serious consequences (3). Transmission of virus from animal to animal is not efficient (4), but both horizontal (5) and vertical (6, 7) transmission of VSV has been demonstrated in insects, indicating that insects probably have an important role in maintaining the VSV reservoir in nature. Humans can be infected with VSV at mucosal surfaces as a result of either close contact with infected animals or accidental exposure in the laboratory (8, 9). The resulting infection may either be subclinical or produce mild flu-like symptoms that typically resolve in 5 to 7 days without complication. Vesicular lesions at the site of infection are rarely seen in humans.

VSV is classified in the *Vesiculovirus* genus within the family *Rhabdoviridae*. The virus particle is bullet shaped (80 nm by 180 nm), comprising a ribonucleoprotein core enveloped by host cell-derived plasma membrane. The genome is a single strand (~11 kb) of negative-sense RNA encoding five major virus proteins in defined transcriptional units (TU). The virus N protein associates closely with genomic RNA, forming the viral nucleocapsid (10, 11). The P (also called NS) and L proteins associate to form the functional viral RNA polymerase, which performs both mRNA transcription and genome replication from the nucleocapsid template (12, 13). The M protein is the most abundant protein in the virus particle, forming a layer between the lipid envelope and the nucleocapsid core. The M protein also has a major role in particle

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budding (14, 15) and regulation of viral transcription (16, 17) and as an agonist of host innate immune responses (18–20). The virus G protein forms trimeric spike-like projections on the particle surface that enable the virus to dock with a host cell receptor(s). Once bound to the receptor, the virus particle is endocytosed. When the endosome reaches a pH of <6.1, the G protein undergoes a conformational change that fuses viral and endosomal membranes, releasing viral nucleocapsid and associated viral RNA polymerase into the cytoplasm and thus initiating the replicative cycle. Ensuing primary transcription of viral mRNA (VmRNA) from the nucleocapsid template has a pronounced 3′-to-5′ polarity (21, 22), leading to expression of all viral proteins, and is followed by replication of genomic RNA through a positive-sense genomic intermediate. Secondary VmRNA transcription then provides an abundant supply of all viral proteins needed for assembly and maturation of virus progeny, which bud from the host plasma membrane.

Negative-strand RNA viruses remained refractive to genetic manipulation until 1994, when a system for the rescue of infectious virus from genomic cDNA was devised for rabies virus (23). Soon after, rescue systems were developed for VSV Indiana (VSIV) and other negative-strand RNA viruses (23–29), allowing directed manipulation of the rVSIV genome for expression of foreign proteins (30). This raised the possibility that recombinant rVSIV could be used as a vaccine vector to immunize against heterologous disease(s). Many aspects of the natural history and replication cycle of VSIV favor this application. VSIV can infect humans, resulting in seroconversion (31) without causing significant disease, and the seroprevalence of VSIV in humans worldwide is very low except in endemic foci, ensuring a naive population for vaccine administration. The small, simple genome organized in discrete TU has facilitated insertion and robust expression of foreign genes (32–35), and a strictly cytoplasmic site of replication that does not involve a DNA intermediate greatly reduces the possibility of viral nucleic acid insertion into the host cell genome. VSIV also can replicate to high titers in approved continuous cell lines, facilitating manufacture of vaccines.

The immunogenicity and potential protective efficacy of rVSIV vaccine vectors for a range of human pathogens have been demonstrated in a series of small-animal and nonhuman primate (NHP) studies (32, 34–39). In one such study, NHPs immunized with rVSIV vectors expressing human immunodeficiency virus type 1 (HIV-1) env and simian immunodeficiency virus (SIV) gag were protected from disease following challenge with pathogenic SIV-HIV (SHIV) (35). Protective, SHIV-specific immune responses were increased when rVSIV and MVA vectors were administered in a heterologous prime-boost regimen (40). However, VSIV and the New Jersey (NJ) serotype of VSV are known to have neurotropic and neurovirulence (NV) properties in young mice (41–44) and can be lethal following direct intracranial (i.c.) inoculation of livestock (45). In view of these findings and an established precedent for NV testing of replication-competent viral vaccines prior to human administration (46–49), the prototypic rVSIV/HIV-1 vaccine vectors were tested in a pilot NHP NV study that was modeled on the NV test used for mumps virus (MuV) vaccine seed production lots (50). The results from this study demonstrated that the prototypic rVSIV/HIV-1 vector and closely related variants were insufficiently attenuated for clinical evaluation (51). Consequently, a variety of different attenuation strategies were developed in an effort to reduce rVSIV vector virulence. These strategies drew on existing methods of attenuation (38, 52–58) but also included novel combinations of specific mutations that were designed to enhance attenuation and produce a range of attenuated variants from which the most ideal vector(s) could be selected for clinical study (59). Murine NV and immunogenicity models were then used to assess vector attenuation and the effect of increasing attenuation on HIV-1-specific immune responses (60).

The work described here extends previous studies of rVSIV attenuation and immunogenicity from mice into NHPs (59, 60). The results of these studies demonstrate successful approaches to attenuation of rVSIV in the NHP central nervous system (CNS). In spite of greatly diminished NV in the NHP CNS, some highly attenuated rVSIV vectors were able to elicit cell-mediated immune (CMI) responses that were similar in magnitude to those induced by the much more virulent prototypic rVSIV vector. These data provide a detailed view of the nature and extent of neurological injury resulting from direct intrathalamic (i.t.) inoculation of NHPs with different attenuated forms of rVSIV and wild-type VSIV (wt VSIV), and they demonstrate that virulence and immune response can be dissociated for some rVSIV vectors. These findings have permitted identification of an rVSIV/HIV-1 vaccine vector that now has been successfully evaluated in a phase I clinical trial.

MATERIALS AND METHODS

Cells and virus. Vero and baby hamster kidney (BHK) cell lines were obtained from the American Type Culture Collection (ATCC) and propagated at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), sodium pyruvate (20 mM), and gentamicin (50 μg/ml). The wt VSV used in this work as a virulence marker in NV studies was the San Juan isolate, Indiana serotype, kindly provided by J. Rose (Yale University, New Haven, CT). All of the attenuated rVSV vectors described for these studies were derived from the rVSV Indiana serotype (27), modified by G gene switching to generate the New Jersey (NJ) serotype (61) where appropriate for immunogenicity studies. Virus propagation in cell culture and virus titration by plaque assay have been described previously (59).

Generation of attenuated rVSV vectors. The generation of rVSVN2CT1, rVSVN3CT1, and rVSVM_{NCP}CT1 vectors containing N gene translocations and M_{NCP} mutations in combination with CT1 G gene truncations have been described previously (59). These vectors were further modified to express HIV-1 strain HXBc2 gag p55 from an extra TU inserted in the first position of the virus genome, generating rVSVN3CT1gag1, rVSVN4CT1gag1, rVSVN4CT9gag1, and rVSVM_{NCP} CT1gag1 (60). Propagation-defective rVSVGstem-gag1 lacking the G gene was generated as described previously (62). The rVSVgag5 vector used in this work as an nonattenuated reference standard during immunogenicity studies was generated by cloning the gag p55 open reading frame into an XhoI/NheI expression cassette located between the virus G and L genes (34). To construct vectors encoding temperature-sensitive (ts) mutations in the N and L genes (rVSVtsN+Lgag1), the N and L genes in vector genomic cDNA were replaced with the corresponding genes from mutant viruses, tsG11(N) and tsG41(L) (63, 64). Briefly, viral RNA was isolated from Vero cells infected with mutant virus at a permissive temperature. N and L gene sequences were then amplified from infected-cell RNA by reverse transcription-PCR (RT-PCR) and cloned back into genomic cDNA. Successful incorporation of N and L genes containing ts mutations was confirmed by nucleotide sequence comparisons with reference virus strains. The ts phenotype of the resulting rVSVtsN+L vector was verified by plaque assay and growth kinetics studies at permissive (32°C), partially restrictive (37°C), and restrictive (39°C) temperatures. The rVSVtsN+L vector was then modified to express the gag gene from

A

rVSV Vectors	Route	Dose (pfu/mL)	No. of Animals	Day of Sacrifice
VSV WT	IT	10^7	3 3	7 21
UV-inactivated VSV WT	IT	10^7	3 3	7 21
rVSV CT1	IT	10^7	3 3	7 21
rVSV N2CT1	IT	10^7	3 3	7 21
rVSV N3CT1	IT	10^7	3 3	7 21
PBS Control	IT	0	3 3	7 21

B

rVSV Vectors	Route	Dose (pfu/mL)	No. of Animals	Day of Sacrifice
VSV WT	IT	10^7	2	7
rVSV M_{NCP}CT1Gag1	IT	10^7	3 3	7 21
rVSV GstemGag1	IT	10^7	3 3	7 21
rVSV tsN+LGag1	IT	10^7	3 3	7 21
rVSV N4CT9Gag1	IT	10^7	3 3	7 21
rVSV N3CT1Gag1	IT	10^7	3 3	7 21
rVSV N4CT1Gag1	IT	10^7	3 3	7 21
UV-inactivated rVSV N4CT1 Gag1	IT	10^7	2 2	7 21

FIG 1 Genetic organization and nomenclature of rVSV vectors. Nomenclature of rVSV vectors reflects major attenuating mutations. NHP inoculation was by the i.t. route for both NV studies 1 and 2 (A and B, respectively).

the first TU in the genome as described for the other vectors. UV-inactivated wt VSV and rVSVN4CT1gag1 were prepared by exposing unshielded virus preparations in phosphate-buffered saline (PBS) to an inactivating dose(s) of UV radiation in a UV Stratalinker (Stratagene Inc.). Inactivated virus preparations were assayed for loss of infectivity following adsorption to Vero cell monolayers and incubation at 32°C for 7 days. The corresponding NJ serotype vectors ($rVSV_{NJ}$) were generated by switching the gene encoding the G_{IN} protein with that encoding the G_{NJ} protein in each case (60, 61). The methods used in this work for recovery of replication-competent rVSV vectors and the propagation-defective rVSVGstem-gag1vector from genomic cDNA using a helper-virus free rescue procedure have been described in detail previously (59). The appearance of cytopathology characteristic of VSV 2 to 7 days posttransfection indicated that rescue had occurred. Rescued virus was then plaque purified on Vero cell monolayers (Vero cells expressing VSV G protein for rVSVGstem-gag1), amplified in either Vero or BHK cells, and purified by centrifugation through a 10% (wt/vol) sucrose cushion. The purified virus pellet was resuspended in PBS (pH 7.0), flash frozen in an ethanol-dry ice bath, and titrated on Vero cell monolayers in preparation for NV and immunogenicity studies.

Intrathalamic inoculation of NHPs. NV studies were performed at Charles River Laboratories, Inc., Preclinical Services (Sparks, Nevada). The cynomolgus macaque (*Macaca fascicularis*) studies described here adhered to the regulations outlined in the USDA Animal Welfare Act (9 CFR, parts 1, 2, and 3) (65) and the conditions specified in the *Guide for the Care and Use of Laboratory Animals* (66). The health of all NHPs used in these studies was monitored twice daily, and any clinical signs of illness or distress were immediately reported to the veterinarian, who promptly recommended either treatment for minor ailments and injuries or euthanasia for profound effects such as severe emesis or convulsions.

The genetic structure and nomenclature of rVSV vectors investigated in the NHP studies described here are shown in Fig. 1. All purified virus stocks prepared for i.t. inoculation of NHPs had the anticipated genome nucleotide sequence, were determined to be free from bacterial and mycoplasma contamination by culture in LB broth and by real-time, quantitative PCR (RT-qPCR) analysis, respectively, and were free of measurable endotoxin(s).

Groups of six 2- to 3.5-year-old cynomolgus macaques, each comprising 3 males and 3 females, were inoculated i.t. with 10^7 PFU of virus in 0.2 ml of PBS per animal. The inoculation procedure has been previously described (51). Briefly, anesthetized animals were injected through two small holes drilled in the skull approximately 1.5 cm on each side of the sagittal suture and 0.5 cm from the coronal suture. Half of the inoculum (0.1 ml) was delivered to the right and half to the left thalamic region by 25-gauge needle. Incisions were closed and animals were returned to their cage following recovery from anesthesia. Non-anti-inflammatory analgesics were given at the discretion of the veterinarian to prevent postsurgical discomfort. Animals were monitored twice daily for clinical signs of disease, including inactivity, dyspraxia, tremors, and weakness. Changes in food consumption, body weight and temperature, serum chemistry, and hematology were also monitored. Prior to necropsy (scheduled or unscheduled), the macaques were exsanguinated under deep anesthesia induced with ketamine and Beuthanasia-D.

Histopathology and measurement of infectious virus and viral RNA in the CNS. At days 7 and 21 postinoculation, 3 animals from each group were necropsied and standard sections of the CNS, including frontal cortex, occipital cortex, thalamus, basal ganglia, cerebellum, and cervical, thoracic, and lumbar spinal cord, were collected and prepared for histological examination as previously described (51). Changes in brain histology profiles were independently scored and peer reviewed by three board-certified veterinary pathologists in blinded fashion for each study. In both NV studies, CNS sections were analyzed for the presence of inflammatory

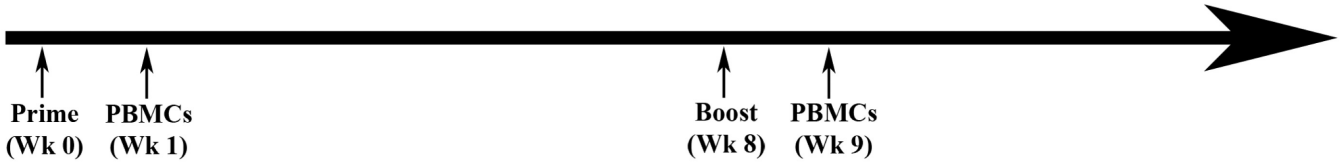
TABLE 1 Study design for rVSV vector immunogenicity in NHPs[a]

A

Group	# of Animals	Prime $rVSV_{IN}$	Boost $rVSV_{NJ}$	Dose (pfu)	Route
1	5	ts N+L	ts N+L	1 x 10^7	IM
2	5	ts N+L	ts N+L	1 x 10^7	IN
3	5	N4CT9	N4CT9	1 x 10^7	IM
4	5	N4CT9	N4CT9	1 x 10^7	IN
5	5	Gag 5	Gag 5	1 x 10^7	IM
6	5	Gag 5	Gag 5	1 x 10^7	IN

B

Group	# of Animals	Prime $rVSV_{IN}$	Boost $rVSV_{NJ}$	Dose (pfu)	Route
1	5	M_{NCP}	M_{NCP}	1 x 10^7	IM
2	5	M_{NCP}	M_{NCP}	1 x 10^7	IN
3	5	N4CT1	N4CT1	1 x 10^7	IM
4	5	N4CT1	N4CT1	1 x 10^7	IN
5	5	G-stem	G-stem	1 x 10^7	IM
6	5	G-stem	G-stem	1 x 10^7	IN
7	5	Gag 5	Gag 5	1 x 10^7	IM
8	5	Gag 5	Gag 5	1 x 10^7	IN



[a] IM, intramuscular; IN, intranasal.

and necrotic lesions. In the first NV study, total lesion severity was scored on a scale of 0 to 4, as follows: 0, no lesions; 1, minimal lesions; 2, mild lesions; 3, moderate lesions; and 4, marked lesions. Given that necrotic lesions are associated with permanent neurological sequelae and are therefore more of a safety concern, necrotic lesions were also separately scored on the same scale in the first NV study. In the second NV study, lesion severity was scored on a scale of 0 to 5 that was slightly different from that used in the first NV study, as follows: 0, no lesions; 1, slight lesions; 2, mild lesions; 3, moderate lesions; 4, marked lesions; and 5, severe lesions. This altered scale allowed greater scoring sensitivity throughout the range of observed lesions, since the vectors under testing were significantly more attenuated than those in the first NV study when evaluated in the mouse NV model, and both inflammatory and necrotic lesion types were separately catalogued.

Levels of infectious virus and viral RNA (vRNA) present in tissue homogenates prepared from CNS sections and cerebrospinal fluid (CSF) were measured by plaque assay and RT-qPCR, respectively, as previously described (51).

Immunization of rhesus macaques. Groups of 5 animals, immunologically naive for VSV and HIV-1 gag protein, were anesthetized with ketamine (5 to 10 mg/kg of body weight) and inoculated intramuscularly (i.m.) with 10^7 PFU of purified virus in 2 ml of PBS (1 ml in each thigh) (Table 1). For intranasal (i.n.) inoculations, 0.4 ml of virus preparation containing 5×10^6 PFU was delivered dropwise to each nostril (0.8 ml containing 10^7 PFU total/macaque) with the anesthetized animal lying flat on a table and with the head tilted back for 2 min after dose delivery. Eight weeks later, animals were boosted by either i.m. or i.n. inoculation with 10^7 PFU of the corresponding NJ serotype switch vector. Blood was drawn at weeks 0, 1, 2, 4, 8, 9, and 12 postinoculation for evaluation of immune responses. gag-specific CMI responses were measured by gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay as previously described (40). For measurement of gag-specific IgG, 96-well Immulon flat-bottom enzyme-linked immunosorbent assay (ELISA) plates (Thermo Labsystems) were coated with HIV-1 gag p24 (20 ng/well) for 18 h at 4°C. Plates were then washed three times with 1× PBS containing 0.1% Tween 20 and blocked with 1× PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween 20 for 2 h at room temperature. Plates were then washed three times, and serum samples diluted 1:64 in 1× PBS and 1% BSA were added to duplicate wells, followed by serial 2-fold dilution across ELISA plates and incubation at room temperature for 1 h. Plates were then washed three times, and goat anti-monkey horseradish peroxidase (HRP)-conjugated antibody (Pierce) diluted 1:10,000 in 1× PBS containing1% BSA was added to each well. Following 1 h of incubation at room temperature, plates were washed three times and color was developed with 3,3′,5,5′-tetramethylbenzidine (Sigma) for 10 min at room temperature, followed by termination of the reaction with 1 N sulfuric acid. Color intensity was read at 450 nm, and endpoint titer was defined as the reciprocal of the last serum dilution resulting in an optical density at 450 nm (OD_{450}) that was equal to the mean plus 3 standard deviations from that of naive sera.

VSV_{IN} neutralizing antibody was assayed at week 3 using a standard neutralization assay (40).

RESULTS

Clinical signs of disease following i.t. inoculation of NHPs. Two independent NHP NV studies were performed. The first study was conducted to determine if the attenuation observed in murine NV studies (59) would also be observed in NHPs. The second NV study included vectors from the first study that were modified to express HIV-1 gag protein.

In both NHP NV studies and as previously observed (51), animals inoculated with wt VSV into the thalamus developed obvious signs of severe neurological disease, including ataxia, tremors, inability to feed, severe weakness, and partial paralysis, by day 6 or 7 postinoculation and were sacrificed accordingly to prevent unnecessary suffering. In the first NV study, one animal receiving wt VSV displayed less severe signs of disease and survived until the end of the study. None of the animals receiving attenuated rVSV vectors, UV-inactivated rVSV, or PBS showed any clinical signs of disease or adverse changes in body chemistry for the duration of the studies.

Levels of infectious virus and vRNA in the CNS. In both NV studies, the levels of infectious virus and vRNA present in different tissue sections of the CNS at days 7 and 21 postinoculation and in CSF at days 3, 7, and 21 were measured to compare replication efficiency, spread, and persistence among the attenuated vectors. Since the day 21 viral genomic RNA levels and levels of infectious virus were not significantly above the limit of detection for their respective assays in the CNS of macaques receiving attenuated vectors, only day 7 data are shown for comparative purposes.

In the first NV study, the effect of combining specific N gene

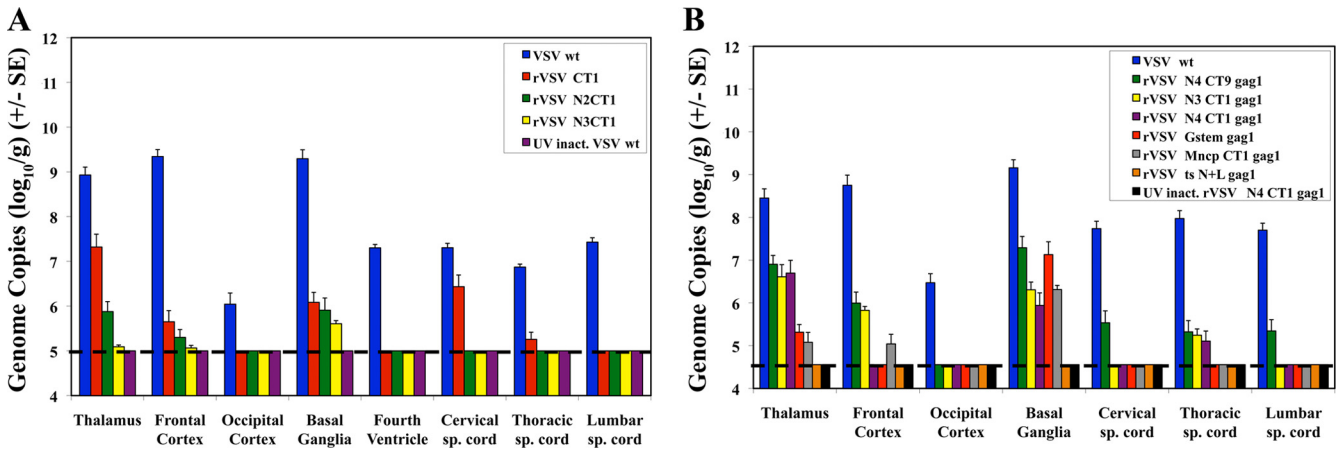


FIG 2 Levels of rVSV genomic RNA detected in CNS sections. Viral genomic RNA detected by RT-qPCR in CNS sections at day 7 postinoculation in NV studies 1 (A) and 2 (B). The detection limits were 1×10^5 and 5×10^4 genome copies/gram of tissue in NV studies 1 and 2, respectively.

translocations and G gene truncations on viral replication in the absence of foreign gene expression was investigated. In animals receiving wt VSV, abundant viral genomes (~10^9 copies/gram of tissue) were detected proximal to the site of inoculation at day 7 (Fig. 2A), persisting at 6×10^6 copies/gram of tissue on day 21 in the only surviving animal. Distal to the site of inoculation, fewer wt VSV genome copies were detected, ranging from 10^6 to 10^7 copies/gram in the occipital cortex and spinal cord regions at day 7, decreasing significantly by day 21 (data not shown). Genome copies of up to 10^6/ml were detected in CSF at day 3 but were absent at day 21. The quantities of infectious virus in animals receiving wt VSV generally reflected levels of virus genome copies detected in the CNS, but at relatively much lower levels. Accordingly, infectious virus was found only at or near the site of inoculation at day 7 (10^4 to 10^6 PFU/gram of tissue) and at day 3 in the CSF of all 6 animals (10^2 to 10^4 PFU/ml) and not in either CNS tissue or CSF at day 21 in the sole surviving animal. In contrast to the case with animals receiving wt VSV, no infectious virus was detected in the CNS or CSF of animals receiving rVSVCT1, rVSVN2CT1, or rVSVN3CT1 when tested at days 3 (CSF only), 7, and 21. As seen for animals inoculated with wt VSV, the levels of rVSVCT1, rVSVN2CT1, and rVSVN3CT1 genomic RNA detected in the CNS at day 7 were greatest proximal to the site of inoculation but were 100- to 1,000-fold lower than levels detected at corresponding sites in animals receiving wt VSV. Viral genomic RNA was detected at low levels (just above the limit of detection) in the CSF of macaques receiving the three attenuated vectors at day 3 postinoculation but not at days 7 and 21. By day 21, viral genomic RNA was not detected in any brain section from animals receiving rVSVN3CT1 and was barely above the level of detection close to the inoculation site in animals receiving rVSVN2CT1. In general and consistent with the degree of attenuation previously observed *in vitro* and in murine NV studies (59, 60), genome copies of the rVSVN2CT1 and rVSVN3CT1 in CNS sections were lower than for rVSVCT1 at day 7, most notably in spinal cord sections.

For the second NV study, rVSVN2CT1, rVSVN3CT1, and other attenuated rVSV vectors were modified to express HIV-1 gag from the first TU in the genome as potential clinical vaccine candidates. Also, since few macaques survived longer than 8 days after inoculation with wt VSV in the first NV study, the second NV study protocol included animals inoculated with wt VSV for analysis at day 7 but not day 21. As in the first NV study, animals receiving wt VSV had moderate to high levels of viral genomic RNA (Fig. 2B) and proportionately lower levels of infectious virus throughout CNS sections at day 7. As in the first NV study, infectious wt VSV was detected in CNS sections at day 7 (1×10^2 to 1×10^6 PFU/gram of tissue) and in CSF at day 3 (2.5×10^2 to 3.9×10^3 PFU/ml), and viral genomic RNA was detected at days 3 and 7 (2.2×10^8 and 5.7×10^6 genome copies/ml, respectively). No infectious virus was detected in CNS sections at days 7 and 21 and in CSF at days 3, 7, and 21 in macaques receiving attenuated vectors, except for 2 animals inoculated with rVSVN4CT9gag1. In both of these animals, infectious virus was barely above the level of detection only at day 7 in sections close to the inoculation site, and it was barely above the level of detection in the spinal cord of one animal (~30 PFU/gram of tissue in each case). All of the attenuated vectors produced ~50- to 1,000-fold-lower levels of viral genomic RNA than wt VSV in corresponding CNS sections at day 7, with highest levels detected proximal to the site of inoculation and either very low levels or no viral genomes detected in distal CNS sections. No genomic RNA was detected in animals receiving any of the attenuated vectors at day 21. No viral RNA was detected in animals receiving UV-inactivated rVSVN4CT1Gag1.

CNS histopathology profiles. In the first NV study, the CNS sections analyzed included the frontal cortex, midbrain (thalamus, basal ganglia, and choroid plexus), cerebellum and brain stem, occipital cortex, and the cervical, thoracic, and lumbar spinal cord. The findings in the CNS for NV study 1 are summarized diagrammatically in Fig. 3. Macaques receiving wt VSV became seriously ill and either died on days 6 to 7 or were sacrificed on schedule at day 7. One animal receiving wt VSV displayed less severe signs of disease and survived until day 21. Therefore, the data for wt VSV include the findings from five macaques at day 7 and only one macaque at day 21. Among all the test articles, these macaques had the highest average scores for necrotic lesions (including the destruction of neurons, glial cells, and oligodendrocytes and loss of ependymal cells), and the individual lesions occurring throughout the CNS were often quite severe. In contrast, macaques receiving the three attenuated rVSV vectors had much

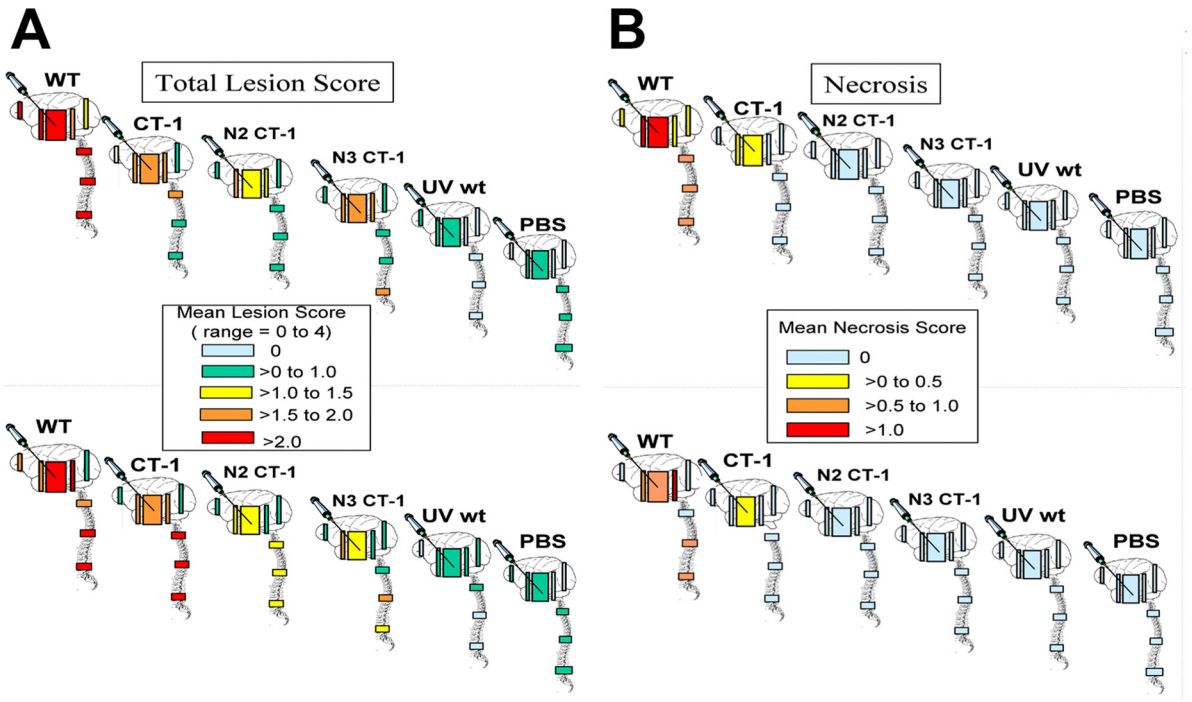


FIG 3 NV study 1: rVSV vector histopathology profiles in the CNS. Panels A and B show relative severities and locations of combined inflammatory and necrotic lesions and necrotic lesions only at days 7 and 21 after inoculation with each rVSV vector.

more limited necrotic lesions that were, on average, very mild and largely restricted to areas proximal to the inoculation site and in the spinal cord, consistent with the high degree of attenuation observed with these vectors in the mouse brain. By day 21, animals receiving rVSVCT1 had necrotic lesions that were generally very mild in brain sections but occasionally moderate to marked in spinal cord sections, while those receiving rVSVN2CT1 and rVSVN3CT1 had only minimal to mild necrotic lesions in the spinal cord and in only occasional brain sections. Macaques receiving PBS and UV-inactivated wt VSV had either no lesions or very slight lesions in only a few brain sections.

Inflammatory responses, typically characterized by perivascular cuffs of mononuclear cells, were greatest at day 7 in animals receiving wt VSV and were, on average, mild to moderate throughout the CNS but marked to severe in some brain and spinal cord sections. At day 7, macaques receiving the three attenuated vectors had, on average, slight to mild inflammatory responses in brain sections and very slight responses in the spinal cord. By day 21, inflammatory responses to rVSVCT1 were notably higher in spinal cord sections (moderate to marked) than they had been day 7. In contrast, day 21 inflammatory responses to rVSVN2CT1 and rVSVN3CT1 were very mild and lower, on average, in brain sections than were those on day 7, although a small increase in inflammatory response was seen in spinal cord sections for both vectors at day 21 relative to day 7. Animals receiving PBS and UV-inactivated wt VSV showed only very minimal inflammatory responses close to the site of inoculation at days 7 and 21.

The vectors tested in the second NV study included further attenuated versions of those tested in the first NV study and some additional vectors attenuated by ts mutations, G gene deletion (G-stem) mutations, and noncytopathic M gene (M_{NCP}) mutations.

The lesion scores in the CNS are represented diagrammatically in Fig. 4. Only two animals received wt VSV; one of these animals developed severe encephalitis and died on day 5, and the other animal was sacrificed on day 7 as scheduled. Both animals displayed mild to moderate necrotic lesions, including neuronal necrosis, and inflammatory lesions throughout the CNS. In contrast, all of the further attenuated rVSV vectors caused minimal necrotic lesions localized mainly proximal to the inoculation site and occasionally in spinal cord sections at days 7 and 21. In these cases, necrotic lesions predominantly involved ependymal cells lining CSF-filled ventricular canals, and there was no clear evidence of neuronal necrosis. Inflammatory responses to the further attenuated vectors at days 7 and 21 were typically slight to mild throughout the CNS and little greater than those produced by UV-inactivated virus. Upon review of all CNS sections from both NV studies and in agreement with mouse NV studies, the attenuated vectors tested in the second NV study were more attenuated, causing less neurological injury than those in the first NV study.

Immune responses to HIV-1 gag. The gag-specific CMI responses elicited by the highly attenuated vectors and the more virulent prototypic rVSVgag5 vector were assessed in rhesus macaques by IFN-γ ELISPOT assay (Fig. 5). Of the attenuated vectors, only rVSVN4CT9gag1 elicited a CMI response equivalent in magnitude to that elicited by rVSVgag5 after i.n. priming. However, following i.m. priming, most of the highly attenuated rVSV vectors elicited a CMI response that was similar in magnitude to that elicited by rVSVgag5 after either i.n. or i.m. inoculation. Interestingly, after i.m. boosting, no significant increase in response above peak postprime levels was observed for any vector except rVSVN4CT1gag1, which had elicited a relatively lower post prime response. Notably, the peak gag-specific CMI response elicited by rVSVN4CT9gag1 and rVSVN4CT1gag1 after i.m. inoculations

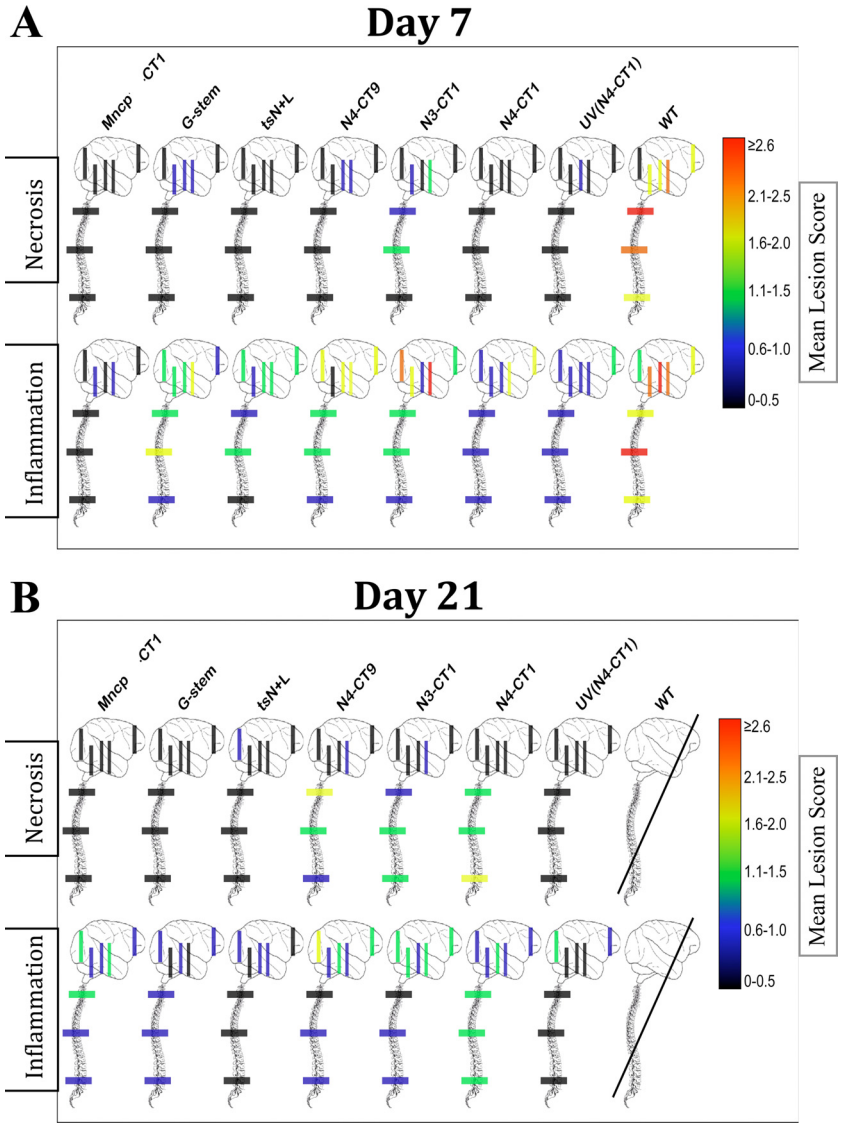


FIG 4 NV study 2: rVSV vector histopathology profiles in the CNS. Panels A and B show relative severities and locations of inflammatory and necrotic lesions at days 7 and 21 postinoculation. Each of the rVSV vectors tested in NV study 2 expressed HIV-1 gag from gene position one.

trended higher than those elicited by the other attenuated vectors, consistent with data from murine studies (60). At 12 weeks postboost, CMI responses had uniformly and significantly decreased but were still above background levels (data not shown).

gag-specific IgG responses were measured to assess the potential of attenuated rVSV vectors to elicit an HIV-1 antigen-specific humoral response (Fig. 6). After i.m. priming at study week 0 and boosting at study week 8, the more virulent prototypic rVSVgag5 vector induced a robust gag-specific humoral immune response. As for the prototypic vector, postboost humoral responses elicited by the attenuated rVSV vectors were higher following i.m. inoculation than after i.n. inoculation, except for tsN+Lgag1, and responses to the rVSVN4CT9gag1 and rVSVN4CT1gag1 vectors trended higher than the others, as seen for the CMI response. Neutralization titers for VSV_{IN} were detected in all but a few animals 22 days postprime, except in those receiving tsN+Lgag1 by the i.n. route, and were highest among the attenuated vectors in animals receiving rVSVN4CT9gag1 (data not shown). Neutralization titers were not determined after boosting with the New Jersey serotype of each vector.

DISCUSSION

Previous work demonstrated that a prototypic rVSV/HIV-1 vaccine vector could elicit protective immune responses in an NHP SHIV challenge model (35, 39), but subsequent studies with this replication-competent vector indicated that it was insufficiently attenuated for evaluation in humans (51). The prototypic vector was subsequently modified by a variety of rational attenuation strategies in an effort to reduce virus virulence (59, 60, 67). Some of the resulting highly attenuated vectors were no longer able to cause disease in a sensitive murine i.c. 50% lethal dose (LD_{50}) model but elicited immune responses in mice that were either equivalent to or greater than those induced by the more virulent prototypic vector (60). In this study, we further investigated the

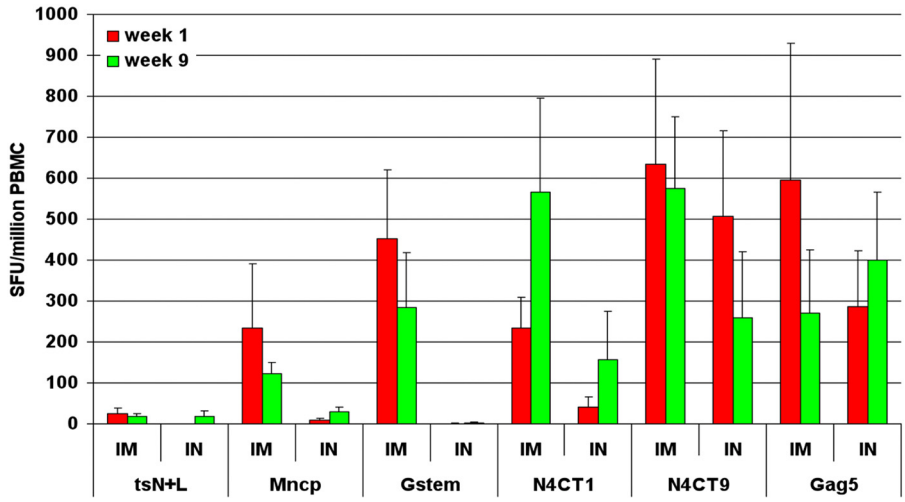


FIG 5 HIV-1 gag-specific cellular immune responses in NHPs. Average gag-specific IFN-γ ELISPOT responses elicited by each rVSV vector following vaccination by the i.m. and i.n. routes are shown with standard errors at 1 week postprime and 1 week postboost (week 9). All vectors expressed HIV-1 gag from the first TU except for Gag5, which was the more virulent prototypic rVSV vector expressing HIV-1 gag from the 5th TU.

relationship between rVSV vector attenuation, neurovirulence, and immunogenicity in NHPs and used the findings to determine an optimal vector for clinical studies.

The first NV study was designed to determine if the synergistic attenuating effect of N gene translocation combined with G protein cytoplasmic tail (CT) truncation demonstrated in a murine i.c. LD_{50} model (59) also resulted in reduced NV in NHPs. This NV study also determined virulence of attenuated rVSV vectors in the absence of foreign (gag) protein expression, a hypothetical situation that could arise in candidate rVSV/HIV-1 vaccine vectors due to gag gene deletion or loss of gag expression by readthrough of transcription stop signals (68). The rVSVN2CT1 and rVSVN3CT1 vectors from the first NV study were later modified to express HIV-1 gag from the first TU in the genome and were tested in the second NV study along with other highly attenuated candidate HIV-1 vaccine vectors, which demonstrated little or no neurotoxicity in the murine i.c. LD_{50} model (60). In both of the NHP studies described here, only wt VSV caused neurological signs of disease following i.t. inoculation, and differences in virulence among attenuated vectors were not clinically apparent, as was the case in the mouse NV model. However, differences in rVSV vector attenuation could be distinguished by histological examination of CNS sections, and these observations were supported by analysis of viral RNA levels in the CNS.

In the first NV study, animals receiving attenuated vectors had significantly lower scores for necrotic and inflammatory lesions in the CNS than animals receiving wt VSV. Among the attenuated vectors, the rVSVN2CT1 and rVSVN3CT1 vectors had lower overall lesion scores and produced less viral genomic RNA in the CNS than the rVSVCT1 vector, consistent with observations made *in vitro* and in mice (59). The most attenuated rVSVN3CT1 vector tested in this study caused a low level of neurological injury in the CNS, consisting of predominantly inflammatory responses, indicating that this vector backbone had a desirable safety profile and would likely be suitable for further vaccine development. To further investigate the NV of these combination mutants, the

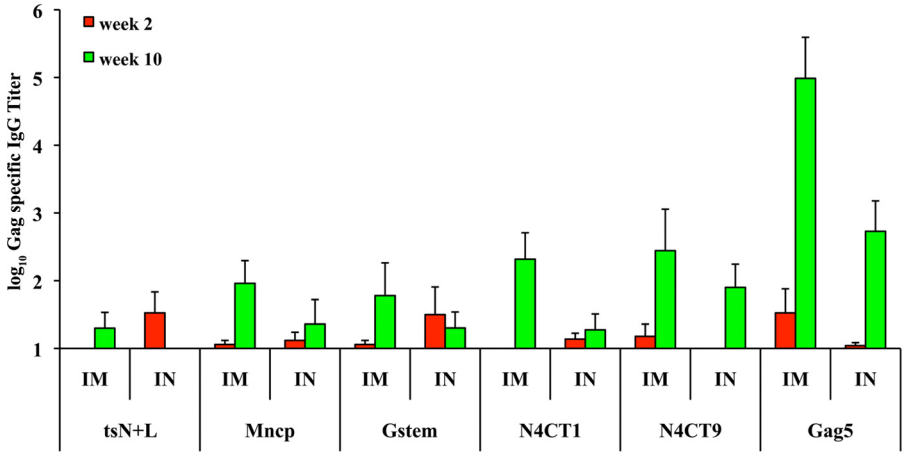


FIG 6 HIV-1 gag-specific humoral immune responses in NHPs. Average gag-specific IgG titers elicited by each rVSV vector after i.m. and i.n. vaccination are shown with standard errors at week 2 postprime and 2 weeks postboost (week 10).

HIV-1 gag gene was added to rVSVN2CT1, rVSVN3CT1, and rVSVN3CT9 as the first TU in the genome, generating rVSVN3CT1gag1, rVSVN4CT1gag1, and rVSVN4CT9gag1, respectively. This maximized expression of gag antigen due to proximity to the 3′ transcription promoter (60, 69) and also increased vector attenuation by translocating all rVSV genes further away from the 3′ transcription promoter (52). The gag gene also was positioned as the first TU in the genomes of rVSVM_{NCP}CT1gag1, rVSVtsN+Lgag1, and rVSVGstem-gag1. All of the resulting attenuated vectors produced greatly reduced pathology in the CNS compared to wt VSV, causing necrotic and inflammatory lesion scores that were not significantly greater than those of UV-inactivated rVSVN4CT1gag1. The rVSVtsN+Lgag1 vector seemed unable to measurably replicate, possibly because NHP body temperature, which typically is 37.6°C, was nonpermissive for this ts mutant. As expected, the propagation-defective rVSVGstem-gag1 vector was also highly restricted for spread throughout the CNS and caused very minimal neuropathology. Interestingly, the presence of rVSVGstem-gag1 mRNA in spinal cord sections (data not shown) demonstrated the potential for mechanical dissemination of all vectors from the inoculum to distal sites during dosing, presumably via the CSF. Among the remaining propagation-competent vectors, the rVSVN4CT1gag1, rVSVN4CT9gag1, and rVSVM_{NCP}CT1gag1 vectors caused the lowest level of neuropathology overall.

The attenuation of vectors containing combinations of N gene translocations and G gene truncations (rVSVN2CT1, rVSVN3CT1, rVSVN3CT1gag1, rVSVN4CT1gag1, and rVSVN4CT9gag1) is due to the following factors: (i) reduced efficiency of G protein transport to the cell surface (70), (ii) likely impaired interaction between the truncated G protein CT (38) and underlying viral core proteins, (iii) limiting nucleocapsid due to reduced levels of N protein synthesis (58), and (iv) reduced secondary transcription, genome replication, viral morphogenesis, and budding resulting from reduced levels of nucleocapsid production (14, 15, 57, 71). The attenuation of rVSVM_{NCP}CT1gag1 relies predominantly on a methionine-to-alanine amino acid change at position 51 in the M protein that prevents the M protein from blocking nuclear export of cellular mRNAs (72), resulting in reduced cell cytopathology *in vitro* and reduced pathogenicity in mice (18–20, 53, 54, 73, 74); combination of the M_{NCP} mutation with the CT1 G protein truncation further enhances attenuation (59). The rVSVG stem-gag1 vector lacks a complete G gene, requiring *trans*-expression of G protein for propagation; therefore, the resulting vector can undergo only a single round of infection *in vivo* (56). The rVSVtsN+Lgag1 vector contains ts mutations in the genes encoding viral nucleocapsid (N) and RNA polymerase (L) proteins, such that *in vitro* replication is significantly reduced at 37°C and ablated at 39°C.

In addition to virus-specific attenuation factors, host factors also contribute to the levels of vector attenuation observed *in vivo*. Chief among these are virus-induced interferons (75, 76), which can strongly inhibit VSV replication in the periphery (77) and probably also in the CNS (78–81). Differences in the ability of attenuated vectors to induce and/or antagonize IFN and differential vector sensitivity to the resulting antiviral effects probably also influence vector virulence and the resulting level of neuropathology observed in the CNS. For example, expression of VSV M gene product(s) likely influences *in vitro* and *in vivo* pathogenicity by downregulation of host cell gene expression, including IFN production (18, 53, 72). Therefore, replication of vectors such as rVSVM_{NCP}CT1gag1, rVSVN4CT1gag1, and rVSVN4CT9gag1, which express either an altered M protein (M_{NCP}) or likely reduced levels of M gene product(s), may be subject to greater control by IFN-mediated pathways and other innate antiviral responses (82).

In spite of significantly reduced virulence, some of the highly attenuated vectors, including the propagation-defective rVSVGstem-gag1, rVSVN4CT1gag1, and rVSVN4CT9gag1, elicited peak gag-specific CMI responses following i.m. inoculation that were equivalent in magnitude to those induced by the much more virulent prototypic rVSV vector. In agreement with previous murine immunogenicity and biodistribution studies (83, 84), these findings indicate that vigorous vector propagation at the inoculation site and spread to surrounding tissues and organs throughout the body are not essential for the induction of robust gag-specific CMI responses following i.m. inoculation of NHPs. Rather, infection of antigen-presenting cells (APC) at the site of inoculation and in draining lymph nodes may be both sufficient and necessary for the induction of robust CMI responses by rVSV vectors. Recent findings support this notion, indicating that a specific subset of APC in the draining lymph node may be largely responsible for induction of the adaptive immune response to VSV in mice (85). In contrast, and except for the more replication-competent N4CT9gag1 vector, most of the attenuated vectors were much less immunogenic by the i.n. route than the prototypic vector, suggesting a requirement for more vigorous virus replication and spread within, and possibly beyond, the nasal epithelium in order to stimulate robust CMI responses in NHPs by this route. Interestingly, there was little overall enhancement in peak gag-specific CMI responses postboost compared to postprime for each rVSV vector (except for rVSVN4CT1gag1, with which the postprime response was very low) by either inoculation route, while gag-specific humoral responses were typically boosted above postprime levels. Since the prime and boost vectors differ only in the surface G proteins, the VSV protein-specific CMI response to other viral proteins may have restricted replication of boosting virus. The use of fully heterologous prime-boost vaccination regimens with rVSV and either plasmid DNA or other viral vectors results in more robust postboost HIV-1-specific cellular immune responses (86–89). The differences in levels of gag-specific immune responses elicited by the attenuated and prototypic vectors tested in this study probably are the outcome of a complex interplay between induction and susceptibility to host innate and acquired immune responses during vector replication and antigen priming of APC.

In conclusion, we have studied the NV and immunogenicity of a range of highly attenuated rVSV vectors in NHPs. The first NV study confirmed previous findings in mice: that rVSV can be rationally attenuated in a stepwise manner by combination of specific G gene truncations and N gene translocations (rVSVN2CT1 and rVSVN3CT1). The second NV study demonstrated that addition of the HIV-1 gag gene as the first TU in the genome of these vectors, generating rVSVN3CT1gag1, rVSVN4CT1gag1, and rVSVN4CT9gag1, further increased vector attenuation. Most of the attenuated vectors in the second NV study caused inflammatory and necrotic lesion scores that were very minimal and not significantly different from those caused by UV-inactivated rVSVN4CT1gag1. Given that there are many similarities between the processes of genomic replication and transcription among

members of the *Mononegavirales*, the combinations of N gene translocations and G gene truncations described here for the attenuation of rVSV may also provide a rational approach for attenuation of many other nonsegmented negative-strand RNA viruses. Importantly, following i.m. inoculation, the rVSVN4CT1gag1, rVSVN4CT9gag1, and rVSVGstem-gag1 vectors elicited gag-specific CMI responses that were similar in magnitude to those induced by the much more virulent prototypic rVSV vector, which afforded protection from AIDS in a SHIV challenge model (39). Of these three vectors, rVSVN4CT1gag1 and rVSVN4CT9gag1 are replication competent and therefore more scalable for vaccine production. rVSVN4CT1gag1 and rVSVN4CT9gag1 demonstrated similar attenuation phenotypes and immunogenicities in NHPs, but previous work showed that rVSVN4CT1gag1 was more attenuated than rVSVN4CT9gag1 in the murine i.c. LD_{50} model (60). Therefore, the rVSVN4CT1gag1 vector was chosen as a candidate HIV-1 vaccine for the first evaluation in humans. A phase I clinical trial of this vector has recently been successfully concluded, and the data will be made available soon.

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